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Characterization and Quantification of Copper Sulfate-Induced Vascularization of the Rabbit Cornea

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A model of angiogenesis in the rabbit cornea, with reproducible onset and duration of responses, was developed by using $CuSO_4$ as the angiogenic stimulus. The vascularization of the cornea was quantified by means of an image analyzer. In addition, the effects of antiinflammatory compounds, dexamethasone and flurbiprofen, on the angiogenic response to $CuSO_4$ were examined. Elvax pellets containing 10–75 μg of $CuSO_4$ implanted in the corneal stroma dose-dependently induced neovascularization, which persisted for more than 64 days at the highest dose. Manual measurements of blood vessel lengths and image analysis measurements of blood vessel areas were comparable during the growth phase of vascularization, but only the image analysis measurements detected a subsequent regression phase. Therefore, the length method of measurement is only useful during the growth phase, whereas the image analysis method is useful during both the growth and regression phases of vascularization. Dexamethasone (50 μg, applied topically, three times a day) and flurbiprofen (100 μg, applied topically, three times a day) suppressed the inflammation produced by corneal implants containing 75 μg $CuSO_4$. However, each drug only inhibited vascular growth by 50% during the 14 days of treatment. Although CuSO4 is not an endogenous angiogenic factor, the model presented in this report may be useful in quantitative evaluation of anti-angiogenic agents. (Am J Pathol 1988, 130:173–178)

ANGIOGENESIS, or growth of new blood vessels, is one of the features of diseases such as cancer, diabetic retinopathy, retrolental fibroplasia, rubeosis, and rheumatoid arthritis. It is also a normal and essential process during embryonic development and wound healing.[1–3] Putative angiogenic factor(s) has been isolated from a variety of tissues, including tumors, retina, corpus luteum, and synovial fluid from arthritic patients.[4–10] Antiangiogenic substances have also been isolated from avascular tissues such as vitreous humor and cartilage.[11–16] Although the precise chemical identity of all of these angiogenic factors is yet to be determined, it is known that they are peptides with a molecular size ranging from 200 to 100,000 daltons. Recently, Fett et al[10] described the chemical characterization of an angiogenic substance isolated from human carcinoma cells, having a molecular weight of about 14,400 daltons, which they called angiogenin. This protein is angiogenic in chick chorioallantoic membrane (CAM) and rabbit cornea in femtomole quantities. A more recent study by Esch et al[17] established the primary structure of bovine pituitary basic fibroblast growth factor, which is highly mitogenic for vascular endothelial and smooth muscle cells *in vitro* and is angiogenic *in vivo*.[17,18]

At present, the measurement of angiogenic factor-induced vascular growth in either the CAM[6] or the cornea[19] is only qualitative. Measurements are more difficult in the CAM because the increase in prolifera-

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tion is determined against the background of developing vasculature. The cornea is preferable as an assay tissue because it is avascular, and any new vascular growth is clearly visible. In the absence of a quantitative measure of neovascularization, comparative assessment of the activity of angiogenic substances from different sources and their inhibitors is not precise.

A model of angiogenesis with reproducible onset and duration of the response is important for the quantitative measurement of vascular growth. The purpose of this study was to develop and quantify a model of angiogenesis with these properties. We have used $CuSO_4$ as the angiogenic agent because angiogenic factors are poorly defined chemically and can only be isolated in extremely small quantities of unknown purity. In addition, the effects of a corticosteroid, dexamethasone, and a nonsteroid antiinflammatory agent, flurbiprofen, were examined on the $CuSO_4$-induced angiogenesis.

Materials and Methods

Male New Zealand white rabbits weighing 2–3 kg were used for all experiments.

Preparation of Elvax 40 Pellets Containing Copper Sulfate

Ethylene vinylacetate co-polymer (Elvax 40, Aldrich Chemical Company) was washed extensively with ethanol before being dissolved in methylene chloride (CH_2Cl_2) to give a final concentration of 12.5% wt/vol, as described by Langer and Folkman.[20]

Finely powdered copper sulfate (Analar $CuSO_4 \cdot 5H_2O$, BDH Chemicals) was then suspended in Elvax solution by shaking, to give final amounts of 10, 25, 50, 75, 100, 120 μg $CuSO_4 \cdot 5H_2O$ per 10 μl of polymer. Ten-microliter drops of the suspensions were pipetted with a positive displacement pipette (Gordon-Keeble) onto sterile slides and were left to dry in a laminar flow cabinet for 1 hour to form pellets 2 mm in diameter, which were then removed with a scalpel blade moistened with 70% ethanol.

Implantation of Elvax pellets

Elvax pellets containing 0, 10, 25, 50, 75, 100, and 120 μg $CuSO_4$ were implanted into rabbit corneas as described by Gimbrone et al.[19] Briefly, groups of rabbits were anethetized with 0.25 ml/kg Hypnorm (0.2 mg fentanyl + 10 mg fluanisone) intramuscularly, followed by sodium pentobarbitone, 6 mg/kg, intravenously.

After instillation of 0.4% benoxinate hydrochloride onto the cornea, a 2-mm tranverse incision, penetrating about halfway through the cornea, was made centrally with a 75L Beaver microsharp blade. An iris spatula (1 mm) was inserted, and a rectangular pocket was formed in the cornea reaching to within 1 mm of the limbus. The Elvax pellets were placed in the pockets, which were closed by gentle pressure with the iris spatula. To prevent infection, 1% Aureomycin ointment was applied to the cornea at the end of the operation.

Stereomicroscopic Observations

In preliminary experiments, the eyes with implants were observed every day for 7 days. Once the pattern, the time of onset, and the rate of vascular growth had been established, observations were made twice in the first week and once in the subsequent weeks with a Zeiss OPM1 operating microscope.

At each observation, the severity of corneal edema and iris vasodilatation were subjectively assessed as mild, moderate, or severe as an indication of the inflammatory response. Black-and-white flash photographs were taken at 1.5× magnification on Pan F film, with a green filter to improve contrast (Kodak Wratten no. 58). Vessel length was measured from 10× life-size enlargements of negatives, with a ruler graduated in millimeters. Because the measurement of lengths of all blood vessels was neither practicable nor feasible, vessel length was defined as the length of the longest vessel or group of vessels. Vessel area, defined as total area of blood vessels, was measured from the negative by a television camera with an input to a Microsight 1 (Digithurst) image analyzer, which measures the areas of black and white on the negative. The areas of blood vessels on the negatives can be directly compared, provided that the negative size and camera magnification are kept constant. The rate and half-life of the regression of the vessel area were also calculated.

Effect of Topical Dexamethasone and Flurbiprofen on the Response to 75 μg $CuSO_4 \cdot 5H_2O$ in Elvax

Elvax pellets containing 75 μg $CuSO_4$ were implanted into rabbit corneas, as described above. The animals were divided into 3 groups. The first group received 50 μg dexamethasone topically, the second group 100 μg flurbiprofen topically, in a volume of 50 μl three times a day from the day of implantation for

14 days. The third group was the control, receiving vehicle only for the same length of time. The eyes with corneal implants were observed and photographed 3, 7, 10, and 14 days after implantation, and neovascularization and inflammation were measured as above.

Results

Postoperative Changes in the Eye

All eyes with control Elvax pellets showed minimal corneal edema and iris vasodilatation after implantation of the pellet.

In contrast, pellets containing 10–100 μg $CuSO_4$ produced dose-related inflammatory responses in the eye within 24 hours of implantation. Levels of $CuSO_4$ up to 50 μg produced mild corneal edema initially, which progressively diminished and disappeared completely by 14 days.

At higher levels of $CuSO_4$, the edema was moderate to severe initially, but diminished to mild edema, which persisted throughout the experimental period. Dilatation of limbal vessels gradually diminished from a moderate or severe level to a mild level which persisted throughout the experiment, at all levels of $CuSO_4$. Iris and conjunctival vasodilatation gradually diminished to zero in a dose-dependent manner over 1–10 days.

In corneas that received control Elvax pellets, postoperative edema lasted for 24 hours, after which the corneas became clear. Figure 1A shows control eyes 1–10 days after implantation of pellets. There was no evidence of blood vessel growth into the cornea for the duration of the experiment.

Corneas receiving pellets containing $CuSO_4$ showed no blood vessel growth until 3 days after implantation, when new vessels could be seen at the periphery of the cornea. Figure 1B shows vascularization 10 days after implantation. At 3 days the vessels have entered the cornea, by 7 days they have reached the pellet, and by 14 days (illustrations not shown) the pellet is completely surrounded by new blood vessels.

Figure 2 demonstrates the change of blood vessel length with time for three different levels of $CuSO_4$. Both maximum vessel length and the time to reach maximum vessel length are dose-dependent. After reaching these maxima, the blood vessels begin to regress. The pattern of regression is such that the shorter vessels in the group regress more rapidly than the longer vessels. Comparison of Figures 2 and 3 shows that although both methods of measurement of neovascularization in response to 75 μg $CuSO_4$ were comparable during the growth phase, only the area measurements clearly detected the regression phase of vascularization.

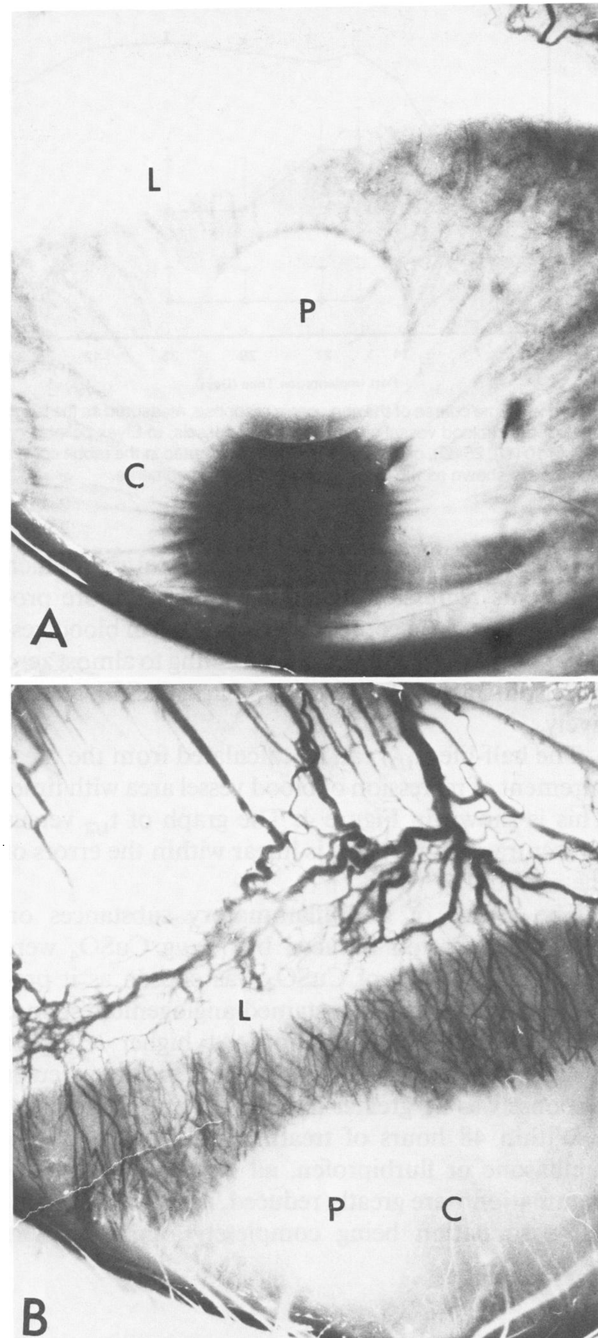

Figure 1A—Reaction of the eye to the Elvax pellet without $CuSO_4$. **B**—Progress of vascularization of the cornea, 10 days after implantation of an Elvax pellet containing 75 μg $CuSO_4$. *L*, limbus; *C*, cornea; *P*, implanted pellet.

Figure 3 shows change in blood vessel area with time for the three levels of $CuSO_4$. Blood vessel areas reach maxima that are dose-related; and the times to reach these maxima are also dose related, though shorter than the time to reach the maximum blood

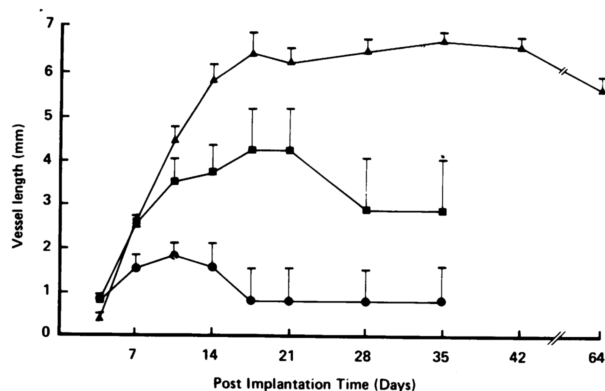

Figure 2—Time course of the angiogenic response, measured as the length of the longest blood vessel or group of blood vessels, to Elvax pellets containing 10 (●), 25 (■), or 75 (▲) μg of $CuSO_4$ implanted in the rabbit cornea. Results are shown as the mean ± SEM of four experiments.

vessel length. The decrease in blood vessel area, which represents regression of blood vessels, is more pronounced than the comparative decrease in blood vessel length, with blood vessel area falling to almost zero by 10 and 35 days for 10 μg and 25 μg $CuSO_4$, respectively.

The half-life ($t_{1/2}$) can be calculated from the measurement of regression of blood vessel area with time. This is shown in Figure 4. The graph of $t_{1/2}$ versus concentration of $CuSO_4$ is linear within the errors of the method.

The effects of antiinflammatory substances on neovascularization induced by 75 μg $CuSO_4$ were studied. This level of $CuSO_4$ was chosen as it produced a uniform and sustained angiogenic response with moderate inflammation. At higher levels of $CuSO_4$ corneal edema was severe, but the neovascular response was no greater than with 75 μg $CuSO_4$.

Within 48 hours of treatment with either dexamethasone or flurbiprofen, all obvious signs of inflammation were greatly reduced, iris and conjunctival vasodilation being completely inhibited. The

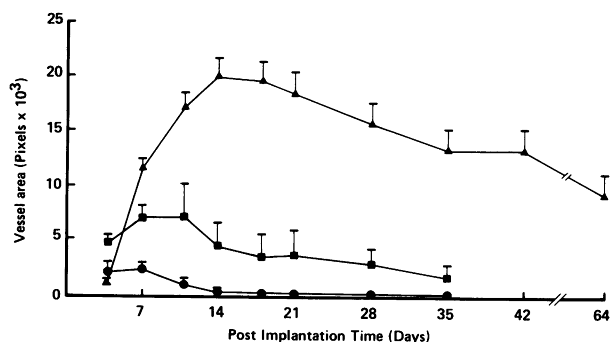

Figure 3—Time course of the angiogenic response, measured as the total area occupied by blood vessels, to Elvax pellets containing 10 (●), 25 (■), or 75 (▲) μg $CuSO_4$ implanted in the cornea. Results are shown as the mean ± SEM of four experiments.

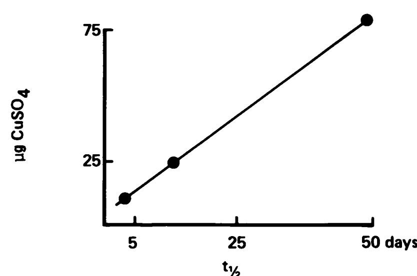

Figure 4—The half-life of regression of the vascular growth plotted against the dose of $CuSO_4$.

dilatation of limbal blood vessels and corneal edema, although noticeably reduced, persisted for the duration of treatment. The effects of treatment with dexamethasone and flurbiprofen on blood vessel length and area are summarized in Figures 5 and 6, respectively. These drugs reduced both length and area of blood vessels significantly. Although most of the visible inflammatory responses to $CuSO_4$ were suppressed, the neovascularization was inhibited by about 50% during the 14 days of treatment. In some experiments in which treatment with dexamethasone was discontinued after 7 days, the growth of blood vessels increased again, reaching control levels by 14 days.

Discussion

In the past, subjective assessment of vessel length has been used to measure neovascularization. The purpose of this investigation was to develop a repro-

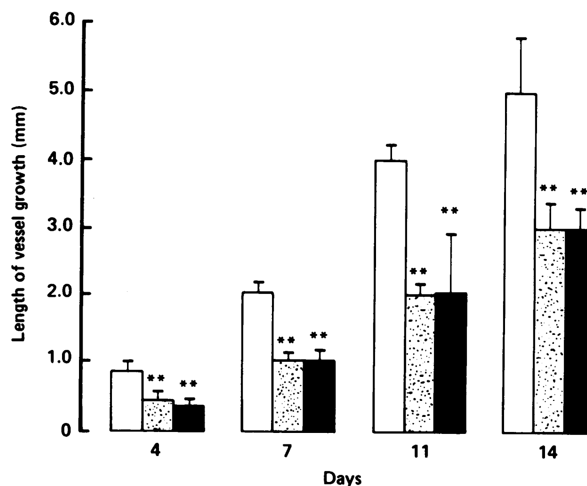

Figure 5—Inhibition of angiogenic response by 50 μg dexamethasone (*hatched column*) or 100 μg flurbiprofen (*solid column*), applied topically to the eye three times a day, after induction of angiogenesis by 75 μg $CuSO_4$ in Elvax pellets. The vessel length was measured as the length of the longest blood vessel or group of blood vessels. The *open column* represents the Elvax pellet only. Results are shown as the mean ± SEM of four experiments. $^*P < 0.05$. $^{**}P < 0.01$.

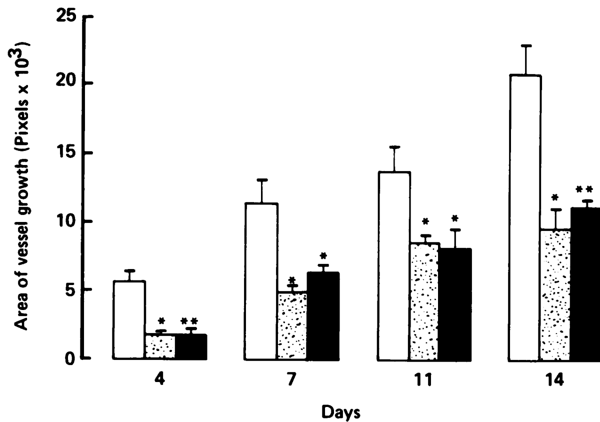

Figure 6—Inhibition of the angiogenic response by 50 μg dexamethasone (*hatched column*) or 100 μg flurbiprofen (*solid column*), applied topically to the eye three times a day, after induction of angiogenesis by 75 μg $CuSO_4$ in Elvax pellets. Vessel area was measured as the total area occupied by blood vessels. The *open column* represents the Elvax pellet only. Results are shown as the mean ± SEM of four experiments. $^*P < 0.05$. $^{**}P < 0.01$.

ducible model of angiogenesis that could be objectively quantified and used for testing antiangiogenic drugs. $CuSO_4$ at levels of 10 μg and above is capable of inducing consistent angiogenesis in the rabbit cornea. The magnitude and duration of response were dose-dependent. Results were quantified by measurement of both vessel length and vessel area from negatives by use of a television camera linked to a Microsight 1 Image analyzer capable of measuring area of black and white on a negative. Of the two dimensions of angiogenesis measured, area is a more representative and precise measurement because it takes into account both long and short vessels. Although our data indicated that during the growth phase area measurements were comparable to length measurements of blood vessels, image analysis detected subsequent blood vessel regression, not apparent by vessel length measurements. Thus, image analysis measurement of blood vessel area is more appropriate than measurement of blood vessel length when the regression phase of vascularization is also investigated.

Lack of a rapid and reliable method to quantify neovascularization has previously made it difficult to accurately determine and compare the potencies of steroidal and nonsteroidal antiinflammatory agents as inhibitors of angiogenesis.[22-24] Dexamethasone and flurbiprofen were tested in our model. Using the above methods, we could accurately assess the inhibitory effects of both drugs on neovascularization; ie, neovascularization was inhibited by approximately 50% by both drugs when tested against 75 μg $CuSO_4$.

In this study, we did not attempt to ascertain the mechanisms whereby $CuSO_4$ induces neovascularization. $CuSO_4$ has been reported to cause both inflammation and neovascularization.[25,26] This accords with our own observations. It is not known whether inflammation is a prerequisite for angiogenesis. Fromer and Klintworth[27-29] have shown that leukocyte infiltration parallels vascular growth and that leukopenic animals do not respond to another angiogenic compound, $AgNO_3$; however, other workers failed to confirm that leukocyte infiltration is necessary.[30,31] In our model, the accompanying inflammatory responses were completely inhibited by dexamethasone and flurbiprofen, while the vascular response was only partly retarded. Because the tissues were not histologically examined for the presence or absence of leukocytes after the treatment with antiinflammatory drugs, we cannot conclusively state whether inflammatory processes are essential for neovascularization. However, the observation that neovascularization is partly inhibited by antiinflammatory drugs suggests a complementary role of inflammatory reactions in the development of neovascularization.

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